Published in final edited form as:

Mol Microbiol. 2015 September ; 97(5): 1006–1020. doi:10.1111/mmi.13083.

GMP synthase is essential for viability and infectivity of *Trypanosoma brucei* despite a redundant purine salvage pathway

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Summary

The causative agent of human African trypanosomiasis, *Trypanosoma brucei*, lacks *de novo* purine biosynthesis and depends on purine salvage from the host. The purine salvage pathway is redundant and contains two routes to guanosine-5'-monophosphate (GMP) formation: conversion from xanthosine-5'-monophosphate (XMP) by GMP synthase (GMPS) or direct salvage of guanine by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). We show recombinant *T. brucei* GMPS efficiently catalyzes GMP formation. Genetic knockout of GMPS in bloodstream parasites led to depletion of guanine nucleotide pools and was lethal. Growth of *gmps* null cells was only rescued by supraphysiological guanine concentrations (100 μ M) or by expression of an extrachromosomal copy of GMPS. Hypoxanthine was a competitive inhibitor of guanine rescue, consistent with a common uptake/metabolic conversion mechanism. In mice, *gmps* null parasites were unable to establish an infection demonstrating that GMPS is essential for virulence and that plasma guanine is insufficient to support parasite purine requirements. These data validate GMPS as a potential therapeutic target for treatment of HAT. The ability to strategically inhibit key metabolic enzymes in the purine pathway unexpectedly bypasses its functional redundancy by

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exploiting both the nature of pathway flux and the limited nutrient environment of the parasite's extracellular niche.

Keywords

Guanine nucleotides; GMP synthase; purine salvage; African sleeping sickness; *Trypanosoma brucei*

Introduction

Trypanosoma brucei is a vector-borne protozoan parasite responsible for human African trypanosomiasis (HAT) (Barrett, 2010, Jacobs *et al.*, 2011, Kennedy, 2013, Stuart *et al.*, 2008). The World Health organization (WHO) estimates that 70 million people in 36 endemic countries in sub-Saharan Africa are at risk, with 10,000 reported deaths per year (WHO, 2013). HAT is caused by *T. brucei* subspecies *T.b. gambiense* and *T.b. rhodesiense*, leading to either chronic or acute infections, respectively. The majority of untreated infections result in death. *T. brucei* is an extracellular parasite that lives in the host bloodstream during the early stage and then migrates to the spinal fluid late in infection. Current therapies depend on identification of both the *T. brucei* subspecies and stage of infection, with late stage disease being the most difficult to treat due to parasite infiltration of the central nervous system. Available therapies are toxic and difficult to administer, putting the infected individuals at greater risk for death. The introduction of nifurtimox-eflornithine combination therapy (NECT) has improved treatment outcomes for late-stage infections (Yun *et al.*, 2010). However, there is still an urgent need to develop more effective and easier to administer therapies to combat HAT.

Trypanosomatids, including *T. brucei*, are unable to synthesize the purine ring *de novo* (Berens *et al.*, 1995, Berg *et al.*, 2010, Boitz *et al.*, 2012, Hammond & Gutteridge, 1984), while human cells are capable of both *de novo* purine synthesis and purine salvage (Zhao *et al.*, 2013). As a consequence, the parasite is auxotrophic for purines and expresses a myriad of purine transporters and salvage enzymes, including 3 phosphoribosyltransferases with overlapping specificities, an adenosine kinase and 6 enzymes that mediate nucleotide interconversion (Fig. 1A). *T. brucei* encodes 12 equilibrative nucleoside transporters that facilitate uptake of purine nucleosides and nucleobases including hypoxanthine, guanine, inosine, and adenine (de Koning *et al.*, 2005, Landfear, 2011, Ortiz *et al.*, 2009, Landfear *et al.*, 2004). Many of the transporters have overlapping activities and stage-specific expression profiles. Salvage pathways are interconvertible and any single purine was shown to be sufficient to support growth *in vitro* (Hammond & Gutteridge, 1984). These data suggested most enzymes in the purine salvage pathway were redundant.

In *Leishmania donovani*, a related parasitic protozoan, genetic knockout studies showed that none of the individual enzymes that convert bases or nucleosides to nucleotides are essential (Boitz *et al.*, 2012). However, there was a preference for salvage of purines via hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and xanthine phosphoribosyl transferase (XPRT) as concurrent knockout of both in promastigote parasites was lethal *in vitro* (Boitz & Ullman, 2006b, Boitz & Ullman, 2006a). Additionally, salvage of adenine or

adenosine was not sufficient to maintain purine nucleoside pools. These results were ascribed to the presence of adenine aminohydrolase activity, which depleted available adenine by converting it to hypoxanthine. These data also suggested the possibility that some of the interconversion enzymes in the purine salvage pathway could be essential for parasite growth. In support, knockout of adenylosuccinate lyase (ADSL) and adenylosuccinate synthetase (ADSS) in *Leishmania* could only be achieved in the presence of an adenine aminohydrolase inhibitor, and the ADSL knockout cells were severely reduced in their ability to infect mice (Boitz *et al.*, 2013).

Unlike *Leishmania* species, *T. brucei* is an extracellular parasite that does not have access to intracellular purine pools. The dominant nucleoside or base precursors in human serum and cerebrospinal fluid (CSF) are hypoxanthine, xanthine and inosine (serum and CSF concentrations are reported to range from 1 - 15 μ M) while other purine bases and nucleosides are much less abundant or undetectable (Hammond & Gutteridge, 1984)(Table 1). These data suggest the possibility that despite the apparent redundancy in the salvage pathway, purine salvage in *T. brucei* will funnel through XMP and inosine monophosphate (IMP) and that some of the interconversion enzymes required to generate GMP and AMP may be essential *in vivo*. Recently it has been shown that RNAi-mediated gene knockdown of ADSS and ADSL in *T. brucei* blood-stage parasites led to reduced growth *in vitro* and attenuated virulence in mice (Mony *et al.*, 2014). Several enzymes in the purine salvage pathway, including guanine monophosphate synthase (GMPS), are highly “druggable targets” (Fig. 1A), as defined by the availability of drug-like inhibitors of homologs from other species (Aguero *et al.*, 2008), making them attractive targets for therapeutic intervention if determined to be essential.

GMPS is an aminotransferase that catalyzes amination of XMP to form GMP, which serves as the precursor for all guanylate nucleotides (Fig. 1B). GMPS links salvaged xanthine, hypoxanthine, adenine, and adenosine via XMP or IMP to GMP formation. GMP is formed in two steps from IMP through the activity of inosine monophosphate dehydrogenase (IMPDH) and GMPS (Fig. 1B). In the opposite direction GMP reductase (GMPR) catalyzes the conversion of GMP back to IMP, allowing interconversion of the guanine and adenine nucleotide pools. In the absence of GMPS, the parasite would be entirely dependent on salvage of guanine/guanosine to maintain GMP pools. Herein we have evaluated the function and essentiality of GMPS in *T. brucei*. Recombinant *Tb*GMPS was purified from *E. coli* for kinetic characterization and was shown to catalyze the formation of GMP from XMP, confirming its enzymatic function. We found that *gmps* null cells could be generated only in the presence of guanine or after expression of a tetracycline (Tet)-regulated ectopic copy of *gmps*. Supraphysiological concentrations of guanine (100 μ M) were required to maintain growth of null cell lines *in vitro*. Removal of guanine supplementation from *gmps* null cells led to reduced levels of intracellular guanine nucleotides, defects in morphology and cell cycle progression, and cell death. The *gmps* null cells were unable to establish an infection in mice, showing that GMPS is also essential for growth of *T. brucei* *in vivo*. These data show that despite the apparent redundancy of purine salvage enzymes in *T. brucei*, the physiological niche (extracellular in blood and CSF) of the parasite does not support *in vivo* growth in the absence of GMPS, a key interconversion enzyme required to supply GMP.

These studies identify GMPS as an attractive target for future drug development in *T. brucei* and show the importance of experimentally assessing gene function despite established dogma.

Results

Recombinant expression and steady-state kinetic analysis of TbGMPS

In order to demonstrate that the *T. brucei* gene annotated as GMPS encodes an active enzyme, recombinant TbGMPS was expressed and purified from *E. coli* to evaluate its catalytic activity. TbGMPS activity was detected using a pyrophosphate-coupled spectrophotometric assay. TbGMPS catalyzed synthesis of GMP with an average k_{cat} of 4.7 s^{-1} (Table 2), which is comparable to the rate published for human GMPS (Nakamura & Lou, 1995). Apparent K_m values were measured for each of the three substrates at fixed concentration of other substrates. $K_{m,\text{app}}$ values for XMP, glutamine and ATP were 8.8, 240, 200 μM , respectively, similar to those reported for other GMPS homologs (Table 2) (Bhat *et al.*, 2008, Lou *et al.*, 1995). Acivicin, which is reported to be a broad-spectrum inhibitor of glutamine amidotransferases, was tested for inhibition of TbGMPS. Acivicin is a glutamate analog and a covalent inhibitor of GMPS from other species (Nakamura *et al.*, 1995, Chittur *et al.*, 2001, Patel *et al.*, 1975). Under the tested assay conditions, Acivicin inhibited *T. brucei* GMPS at mid-micromolar concentrations (apparent $\text{IC}_{50} = 26 \mu\text{M}$) (Table 3).

TbGMPS is essential for growth of *T. brucei* bloodstream form (BSF) cells

To evaluate the essentiality of TbGMPS in *T. brucei* we attempted to generate a *gmps* null cell line by knockout of both *gmps* alleles (*T. brucei* is a diploid organism) in BSF single marker (SM) cells. Parasites were grown in HMI-11 medium in the presence of 100 μM guanine or guanosine, which were expected to allow rescue of the *gmps* (-/-) knockout by supplementing GMP pools through the activity of HGPRT (Fig. 1). However, we were unable to generate a null cell line under these conditions suggesting that TbGMPS is an essential enzyme. To further evaluate essentiality we generated a conditional null (c-null) cell line. Due to difficulties in cloning *T. brucei gmps* into the pLew100v5 expression vector in the sense orientation, the *Leishmania major gmps* gene was used to generate a Tet-regulated ectopic expression plasmid that was integrated into the genome of SM cells. Upon induction of *LmGMPS* expression with Tet, both *T. brucei gmps* alleles could be replaced by selectable markers, leading to the generation of the TbGMPS c-null *gmps* cells. The c-null cell line was validated by PCR analysis, demonstrating that the *gmps* gene had been removed from both original loci. Depletion of *LmGMPS* from c-null cells by removal of Tet led to a severe attenuation of cell growth, which correlated with a loss in detectable *L. major gmps* transcript levels (Fig. 2A, B). Although very few cells were visible by day 4, cell growth resumed on day 6. Analysis of the surviving cells by qPCR revealed re-expression of the *L. major gmps* transcript (Fig. 2B). Presumably, the escaped cells bypassed Tet-repression, a phenomenon that has been commonly observed in *T. brucei* regulated expression systems (Krieger *et al.*, 2000, Roper *et al.*, 2002, Pratt *et al.*, 2014). The finding that GMPS is essential differs from results obtained in a genome-wide RNAi screen where GMPS was not found to be essential (Alsford *et al.*, 2011), likely due to insufficient knockdown of GMPS by RNAi.

Supraphysiological concentrations of hypoxanthine impairs guanine rescue

We attempted to rescue the growth defect observed in the *TbGMPS* c-null cells by the addition of exogenous guanine. Normal human plasma has been reported to contain less than 1 μM guanosine/guanine (Table 1), however, in standard *T. brucei* culture medium (HMI-11 medium), guanine concentrations up to 100 μM were not capable of fully rescuing growth (Fig. 2A). HMI-11 medium contains 1 mM hypoxanthine, significantly surpassing human plasma and CSF concentrations, which are reported to be on the order of 5 - 10 μM (Table 1). *T. brucei* BSFs possess two purine transporters, H2 and H3 and it was shown that both hypoxanthine and guanine compete for the H2 transporter and both bases are converted to nucleotides by a common enzyme (HGPRT) (de Koning & Jarvis, 1997, de Koning et al., 2005). To address the possibility that high concentrations of hypoxanthine were inhibiting transport and enzymatic conversion of guanine to GMP, hypoxanthine was removed from HMI-11 medium and we generated a new medium (designated HMI-19) that contained no added hypoxanthine or thymidine. However, this medium still contains 10% FBS, which is expected to lead to physiological levels of hypoxanthine (5 – 10 μM range), similar to that recently reported for a minimal *T. brucei* culture medium (CMM) supplemented with 10% FBS but not with added hypoxanthine (Creek *et al.*, 2013) (Table 1). The *gmps* c-null cells grew at similar rates in HMI-11 and HMI-19 media in the presence of Tet. Upon removal of Tet cell growth arrest was once again observed in HMI-19 medium, but exogenous guanine concentrations of 30 μM and 100 μM were now able to fully support growth in this medium, while 10 μM guanine remained insufficient (Fig. 2C). Titration of the effective guanine concentration that is required for rescue was performed, and showed that the effective dose of 50% activation (ED_{50}) is 20 (19 - 22) μM in HMI-19 medium (Fig. 2D). Additionally, adding back hypoxanthine recapitulates the growth defect observed with HMI-11 medium (Fig. 2D). Hypoxanthine concentrations exceeding 60 μM prevented rescue with 100 μM guanine, with an inhibitory ED_{50} = 100 (95 – 112) μM hypoxanthine (Fig. 2E).

GMPS null cells are auxotrophic for guanine and require supraphysiological levels for survival

With the knowledge that excessive hypoxanthine had prevented efficient guanine rescue of *gmps* knockdown, we were able to generate the null cell line using the newly formulated medium (HMI-19) supplemented with 100 μM guanine. Replacement of both *gmps* alleles by the selectable markers, *bsdR* and *hygR*, was confirmed by PCR. Western blot analysis for *TbGMPS* further confirmed the complete absence of the protein (Fig. 3A). The ability to generate *gmps* null cell line avoids the phenomenon of Tet control escape that we observed in the c-null cell line, allowing for a more robust analysis of the essentiality of *TbGMPS*. Upon removal of exogenous guanine from the medium *gmps* null cells rapidly stopped growing and complete death of the culture was observed by day 2-3 (Fig. 3A). We next supplemented cultures with a range of exogenous guanine concentrations (10 – 100 μM) to determine the minimum level needed to sustain growth (Fig. 3A). Guanine levels of 100 μM were required to fully rescue the growth defect caused by *TbGMPS* knockout. Guanine at 10 μM showed minimal to no ability to support growth, while guanine levels of 30 μM provided partial rescue but cells still eventually died with a 3-4 day lag time compared to cells supplemented with 100 μM guanine. As observed with the c-null cells, xanthine,

adenosine, or hypoxanthine at 100 μ M were not capable of rescuing growth of the *gmpr* null cells. These data show that guanine levels must be significantly higher than reported guanine plasma levels and $\sim$ 20 fold higher than hypoxanthine levels before they are sufficient to support cell growth.

Cell cycle and morphologic analysis of c-null and null GMPS cells

To evaluate the effect of GMPS deficiency on the cell cycle, flow cytometry was performed. Cell cycle analysis of the GMPS null cells before (Fig. 4A) and 24 h after the removal of guanine (100 μ M) (Fig. 4B) showed that loss of guanine led to a marked reduction (64%) in the fraction of cells in S-phase and an accumulation of cells in G2/M. These results are consistent with the requirement for guanine nucleotides for DNA synthesis. Similar results were also observed for the c-null cell line 48 h after Tet removal (Fig. 4D and 4E) but the reduction in S-phase was less pronounced and the accumulation of cells in G2/M was greater. The finding of a more robust block of DNA synthesis in the null cell line after guanine was removed is consistent with the observation that the GMPS null cells die more quickly than the c-null cells once the source of guanine nucleotides is removed. Cells were also examined by microscopy after DAPI staining, revealing significant morphological defects after either removal of guanine (null cells) or Tet (c-null cells). Null cells were badly misshapen, have missing or detached flagella and an abnormal texture just 24 h after guanine removal (Fig. 4C). A similar result was observed for the c-null cells 48 h after Tet withdrawal, where *Lm*GMPS knockdown resulted in short and enlarged cell bodies with absent or complex flagella configurations (Fig. 4F). DAPI staining was used to analyze the distribution of nuclei and kinetoplast in the c-null cells. An increase in 2K2N (14%) and 1K2N (30%) cellular bodies was observed consistent with arrest in the G2/M phase (Fig. S1).

GMPS is essential for virulence of *T. brucei* in mice

To address the question of whether *in vivo* host plasma levels are sufficient to bypass the metabolic block imposed by loss of *Tb*GMPS, we determined if *gmpr* null cells were able to establish an infection in mice. Mice were injected with either *gmpr* null or SM BSF (control) cells and the course of infection was monitored over a period of 30 days. All SM-infected mice developed detectable levels of parasitemia by day 3 post-infection, with all mice dying (or being euthanized) with hyperparasitemia by day 6 (median survival was 5.5 days post-infection) (Fig. 3B). In contrast, the *gmpr* null infected mice never showed detectable levels of parasites in their blood and all animals survived until the end of the study (day 30 post-infection) (Figs. 3B and 3C). These data demonstrate that mouse plasma does not contain sufficient levels of guanine or guanosine to support parasite growth in the absence of functional GMPS expression.

GMPS inhibitors block parasite growth

To determine if the known GMPS inhibitors Acivicin and 6-diazo-5-oxo-L-norleucine (DON) also inhibited parasite growth we cultured wild-type parasites in their presence. Similarly to Acivicin, DON has been reported to be a covalent inhibitor of GMPS (Nakamura et al., 1995, Chittur et al., 2001, Patel et al., 1975). Both compounds killed wild-

type (SM) cells with an EC₅₀ in the low micromolar range (1 – 3 μ M)(Table 3). To determine if this killing was on target and caused by inhibition of *Tb*GMPS, we also evaluated these compounds on *gmps* null cells cultivated in the presence of 100 μ M guanine. Both compounds also killed *gmps* null cells with similar dose response to the SM cells, demonstrating that for these compounds GMPS is not the primary target of cell killing. These compounds have also been shown to have off-target cell killing mechanisms in Leukemia L1210 cells (Lyons *et al.*, 1990) and in *C. albicans* (Rodriguez-Suarez *et al.*, 2007).

GMPS knockdown leads to reduction in guanine and adenosine nucleotide pools

To analyze the effects of *gmps* knockout on cell nucleotide pools, nucleotide levels were measured by LC-MS/MS analysis. In the absence of guanine supplementation, rapid death of *gmps* null cells prevented collection of sufficient quantities of cells for nucleotide analysis so the *gmps* c-null cell line was evaluated instead. The *gmps* c-null cells were grown $\pm$ Tet in HMI-19 medium for 2 days and cells were collected and processed for analysis. Attempts to collect cells past day 2 after Tet removal failed because cells lysed upon centrifugation, indicating that by day 3 (-Tet) the cell integrity was already significantly compromised. Intracellular guanine nucleotide pools (GMP, GDP and GTP) were reduced 50 - 75% by day 2 (-Tet) relative to control (+Tet) cells consistent with loss of GMPS expression (Fig. 5). In addition AMP and ADP levels were reduced by 30 – 50%, respectively, and IMP decreased by 40% (Figs. 5 and S2 A, B). XMP levels increased by 22%, but this change did not reach statistical significance (Figs. 5C and S2C). ATP levels were also monitored, but due to the large number of ATP hydrolyzing enzymes, methods that require significant cell manipulation can result in erroneous results. To avoid this issue we used CellTiter-Glo assays to assess ATP levels as the reagent can be added directly to cell cultures reducing the error of the measurement. In this assay the level of luminescence is directly proportional to the amount of ATP. We showed that the assay was linearly dependent on the number of cells and then we measured luminescence for a quantified number of cells (500,000) based on counting using a hemocytometer. We did not observe a significant change in ATP pools after Tet removal (Fig. S2D).

Comparison of human and *T. brucei* GMPS binding sites

We considered the potential for identification of species-specific inhibitors of *T. brucei* GMPS that will not inhibit the human enzyme. While an X-ray structure of *T. brucei* GMPS is not available, GMPS structures for both human and *E. coli* GMPS have been solved bound to various ligands (e.g. XMP, AMP and citrate). We aligned the amino acid sequences of *T. brucei* GMPS with the human and *E. coli* enzymes (Fig. S3A) and then compared the residues in the various nucleotide and citrate binding sites (Figs. S3B and S3C). Residues were identified in all three binding sites that differ between the human and *T. brucei* GMPS suggesting it may be possible to identify species selective inhibitors.

Discussion

Trypanosomatids including *T. brucei* are unable to synthesize purines *de novo* and rely on uptake and purine salvage pathways to acquire these essential nutrients. Because of the

redundant nature of these salvage pathways, which support the interconversion of guanine and adenine nucleotide pools, it was thought that no single enzyme in the pathway would be essential for parasite growth. Herein we show that recombinant *T. brucei* GMPS efficiently catalyzes synthesis of GMP, and that *gmps* null bloodstream form parasites are auxotrophic for guanine. Importantly, we also show that the guanine concentrations required to support growth of the *gmps* null cell line *in vitro* (100 μ M) significantly exceed, not only physiological levels of guanine in human plasma (guanine/guanosine human serum < 1 μ M), but the levels of any salvageable nucleosides or bases in human plasma and CSF (Table 1). As a result, GMPS is essential for the infectivity of *T. brucei* in a mouse model and would be expected to be essential to sustain a human infection. The predominant purine bases and nucleosides available for salvage in both mammalian plasma and CSF are reported to be hypoxanthine and xanthine (Boulieu *et al.*, 1983, Hartwick *et al.*, 1979, Slowiaczek & Tattersall, 1982). Thus despite the redundant pathway, the infective blood stage form of the parasite obtains purines primarily through salvage of hypoxanthine and xanthine via HGPRT and XPRT, leaving the parasite vulnerable to inhibition of key interconversion enzymes like GMPS. Taken together with the high druggability score for GMPS (0.6) and species differences in the binding pocket, these data suggest that GMPS will be an excellent target for future drug discovery efforts against *T. brucei*.

The purine salvage pathway may offer significant promise for therapeutic intervention against a range of pathogenic protozoa and fungi enzymatic steps. In addition to our results showing that GMPS is essential in *T. brucei*, studies using RNAi-based gene knockdown have shown that both ADSS and ADSL knockdown led to attenuated virulence of *T. brucei* in mice (Mony *et al.*, 2014). The fact that parasites were not fully cleared in that study may be due to the leakiness of the RNAi system, in comparison to our GMPS study which characterized a null cell line. These data suggest that similarly to our findings with guanine, insufficient adenine may be present in serum to support parasite growth in the absence of ADSS and ADSL. Thus, the extracellular niche of *T. brucei* is likely to be a significant contributing factor to the vulnerability of the parasite to disruption of key interconversion enzymes required for the synthesis of GMP and AMP. In the pathogenic fungi *Candida albicans*, knockout of GMPS also led to guanine auxotrophy and to reduced virulence in a systemic candidiasis murine model where availability of plasma guanine was insufficient to support growth (Jiang *et al.*, 2010). In *L. donovani*, ADSS and ADSL knockout cell lines showed reduced virulence (Boitz *et al.*, 2013) despite the fact that *Leishmania* species have access to intracellular purine pools. These results were ascribed to the presence of adenine aminohydrolase in *L. donovani*, which forces the parasite to rely on xanthine and hypoxanthine to supply required purines. While *T. brucei* lacks adenine aminohydrolase, apparently plasma adenine levels are insufficient to support normal growth. Thus despite initial dogma that the purine salvage pathway enzymes would not be essential, current data strongly supports the conclusion that several enzymes in the pathway are essential in both *T. brucei* and *Leishmania*, findings which may likely extend to *T. cruzi* as well.

In *T. brucei*, several additional factors may also contribute to the observed insufficiency of plasma guanine pools to support growth and *in vivo* infectivity of *gmps* null cells. Firstly, *T. brucei* encodes guanine deaminase (Fig. 1A), which would contribute to depletion of any

available environmental guanine and to the funneling of purine salvage through XMP. Secondly, the presence of GMPS may also contribute to depletion of any synthesized GMP leading first to formation of IMP and then to draining of guanine nucleotide pools into either adenine nucleotides via ADSS or into XMP via IMPDH (Fig. 1A). The “dead-end” accumulation of XMP would be the expected consequence of its continued synthesis (IMPDH and XPRT) in the absence of GMPS activity. In support, XMP levels were modestly increased after GMPS knockdown. Finally, competition between hypoxanthine and guanine for both uptake and for conversion to nucleotides by HGPRT may also play a role in preventing utilization of guanine given the significantly higher concentrations of hypoxanthine compared to guanine/guanosine in human plasma. Our data demonstrate that hypoxanthine competes with guanine, reducing or preventing the ability of guanine to rescue the cell growth defect of *gmps* c-null cells. Our studies also highlight the importance of evaluating knockout strains in physiologically relevant medium and they confirm prior results (Creek et al., 2013) that the high levels of hypoxanthine in standard HMI-11 medium are unnecessary to support growth. Excessive hypoxanthine levels were indeed detrimental to our ability to generate the *gmps* null cell line.

Metabolite analysis of purine nucleotide levels showed that the predominate effects of GMPS depletion were on the guanine and adenine nucleotide pools. Guanine nucleotide concentrations were uniformly reduced, a finding that would be a direct consequence of GMPS knockdown. AMP and ADP levels were also lower. Additionally we observed an increase in XMP pools, suggesting that some buildup of this dead-end product occurs as a consequence of the genetic block in degradation. It is important to note that we were only able to conduct nucleotide analysis relatively soon after Tet removal when *gmps* c-null cells were only just entering growth arrest. It is likely that changes in the nucleotide pools were more pronounced further into the growth curve (day 4 –Tet for the c-null cells) and in *gmps* null cells after guanine was removed. An unexplored question is whether or not *T. brucei* mounts an adaptive response to purine depletion as observed with *Leishmania* (Martin et al., 2014), which might contribute to the observed balance of adenine nucleotides relative to XMP and IMP after GMPS depletion.

In conclusion, we have demonstrated that despite having a redundant salvage pathway for the biosynthesis of guanine nucleotides, *T. brucei* parasites require GMPS activity for normal cell growth and infectivity *in vivo*. *T. brucei gmps* null cell lines are auxotrophic for guanine and levels of guanine required to restore growth significantly exceed physiological levels found in human extracellular fluids where parasites live. Our data suggest that similarly to *Leishmania*, the primary purine salvage route in *T. brucei* is through uptake and incorporation of hypoxanthine and xanthine into guanine and adenine nucleotides. GMPS is a highly druggable target and analysis of active site differences with human GMPS suggest that selective inhibitors of GMPS might be identified. Our results suggest that GMPS could be an excellent target for future drug discovery efforts directed at controlling African sleeping sickness.

Experimental Procedures

Ethics Statement

All animal care and experimental procedures were in accordance with University of Texas Southwestern Medical Center (UTSW) IACUC guidelines. Animal experiments were approved by the Ethical Review Committee at the University of Southwestern Medical Center and performed under the IACUC-2012-0012 protocol. All mice were housed in an animal facility with barrier SPF conditions.

TriTrypDB gene accession numbers

GMPS, Tb927.7.2100; IMPDH, Tb927.10.16120; GMPR, Tb927.5.2080; ADSS, Tb927.11.3650; ADSL, Tb927.9.7550; HGPRT, Tb927.10.1470, Tb927.10.1400; XPRT, Tb927.10.1390; APRT, Tb927.7.1780, Tb927.7.1790; AD, Tb927.9.7180, Tb927.9.9740, Tb927.11.3850, Tb927.11.15750; NH, Tb927.3.2960; GDA, Tb927.5.4560; dihydroorotate dehydrogenase (DHODH), Tb927.5.3830 and telomerase reverse transcriptase (TERT), Tb927.11.10190.

GMPS protein expression, purification and assay

GMPS expression and purification—The full-length, codon-optimized *gmgs* gene was synthesized by Genscript® and cloned into the customized pET28 (TEV) vector (Deng *et al.*, 2009) using *Bam*HI and *Hind*III restriction sites to generate an N-terminal His₆-tag fusion protein. The GMPS-pET28 (TEV) construct was transformed into phage-resistant *E. coli* (Novagen). Cultures (2 L) were grown at 37°C to OD₆₀₀ 0.6 - 0.8 and *Tb*GMPS expression was induced by the addition of 200 µM IPTG (isopropylthio-β-galactoside). Cells were incubated overnight at 20°C, harvested by centrifugation and then resuspended in lysis buffer (150 mM NaCl, 50 mM HEPES pH 8.0, 10% (v/v) glycerol, and 10 mM imidazole) supplemented with protease inhibitors (PICI, 1 mg/mL leupeptin 2 mg/ml antipain and 1 mg/mL leupeptin 10 mg/ml benzamidine; PICII, 1 mg/ml pepstatin, 1 mg/ml chymostatin (Sigma)). Cells were lysed by cell disruptor (pressure of 10000-15000 p.s.i.), cell debris was removed by centrifugation and supernatants were applied to a Ni⁺²- column (GE Healthcare Life Science) equilibrated in buffer A (150 mM NaCl, 50 mM Hepes pH 8.0, 10 % (v/v) glycerol, and 10 mM imidazole). *Tb*GMPS was eluted using a gradient from 10 – 100 % buffer B (buffer A plus 1 M imidazole). DTT (10 mM) was added to the fractions containing the eluted protein, which were pooled together and concentrated to 40 mg/mL (30 kDa MWCO Millipore). Protein concentration was determined by Bradford assay (Bradford, 1976). *Tb*GMPS purity was >95% based on SDS-PAGE analysis (Fig. S4).

Steady-state kinetic analysis of GMPS activity—Activity of *Tb*GMPS was monitored using a coupled spectrophotometric assay based on PP_i-dependent phosphofructokinase (Smyth & Black, 1984). Reaction rates were monitored by following oxidation of NADH at 340 nm ($\epsilon = 6220 \text{ M}^{-1}\text{cm}^{-1}$). The standard assay reaction consisted of buffer (90 mM Tris HCl pH 8.5, 10 nM GMPS, 20 mM MgCl₂, and 0.1 mM DTT), 2 mM ATP, 5 mM glutamine, 0.10 mM xanthosine monophosphate (XMP), 0.066 ml pyrophosphate reagent (Sigma P7275) and recombinant *T. brucei* GMPS (10 nM)(linearity confirmed from 4 - 64 nM) in a reaction volume of 0.2 ml. The reaction was performed in

Greiner 96 area flat bottom plates (Phenix Research) at RT and monitored continuously over time (0 – 20 min) on a BioTek Synergy H1 hybrid reader spectrophotometer. To determine the steady state kinetic constants, the concentration of one substrate (XMP 3.8 - 180 μ M, ATP 25 - 1200 μ M, glutamine 50 - 2000 μ M) was varied while keeping the other two at saturating concentrations. Fixed substrate concentrations were: XMP, 0.15 mM; ATP, 2 mM; and glutamine, 5 mM. Data were collected in triplicate and fitted to the Michaelis-Menten equation ($Y = V_{\max} * X / (K_m + X)$) using GraphPad Prism (San Diego). For determination of Acivicin (Sigma) inhibition constants (IC_{50}), inhibitor concentrations were varied in a 3-fold dilution series (1 – 300 μ M) using the standard reaction conditions (above). Assay time was 10 min. Data were collected in triplicate and were fitted by GraphPad Prism using the log(inhibitor) vs. response – variable slope (four parameters) equation.

T. brucei cultivation and generation of transgenic cell lines

T. brucei growth and transfection methods—All studies were done using *T. brucei* bloodstream form (BSF) single marker cells (SM) engineered to express T7 RNA polymerase and tetracycline (Tet) repressor (Wirtz *et al.*, 1999). Cell lines were maintained at exponential growth (10^5 - 10^6 cells/ml) in HMI-11 (Hirumi & Hirumi, 1989) or HMI-19 (Table 1) medium supplemented with 10% fetal bovine serum (FBS) (Tet minus certified) at 37°C under 5% CO₂. HMI-19 medium has the same composition as HMI-11 media except it was modified by the omission of hypoxanthine and thymidine. SM cells were grown in G418 (2.5 μ g ml⁻¹) to maintain the TetR and T7 polymerase inserts. Transfections were performed as described (Burkard *et al.*, 2007) using Not I-linearized DNA (5 μ g) with an Amaxa Nucleofector II followed by selection with the appropriate antibiotic. Antibiotic concentrations were: 2.5 μ g ml⁻¹ G418 (Life Technology), 2.5 μ g ml⁻¹ phleomycin (InvivoGen), 2 μ g ml⁻¹ blasticidin S (Sigma), 1 μ g ml⁻¹ hygromycin B (Sigma), 0.1 μ g ml⁻¹ puromycin (Sigma) and 1 μ g ml⁻¹ Tet (RPI). Where indicated, nucleosides or bases, guanine (Sigma-Aldrich), guanosine (Sigma-Aldrich), hypoxanthine (Sigma-Aldrich), xanthine (Sigma-Aldrich), or adenine (Sigma-Aldrich) were added exogenously to the medium (concentrations ranging from 0.01 – 1 mM). GMPS c-null cells were cultured in Tet (1 μ g ml⁻¹ daily) to maintain expression of the ectopic copy of the *gmps* gene plus appropriate antibiotics in either HMI-11 or HMI-19 media as indicated in the figure legends. *TbGMPS* null cells were maintained by the addition of exogenous guanine (100 μ M) to the HMI-19 culture medium. To determine the effects of loss of GMPS expression on *T. brucei* growth, cells were washed and plated in Tet (c-null) or guanine (null) free medium without antibiotics and cell growth rate was monitored over time. Cell density was determined by counting with a hemocytometer (Bright-Line). Cells number was plotted versus time and total cell numbers were calculated by multiplying cell density by the dilution factor and volume (Wang *et al.*, 2000). The lower limit of detection is 10⁴ cells/ml by this method.

Cell growth effects of GMPS inhibitors—Growth inhibition by Acivicin and 6-diazo-5-oxo-L-norleucine (DON) (Sigma) (0.01 μ M – 33 μ M) was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Cells were grown in 96 well CellStar plates (Greiner) with inhibitor in HMI-19 medium (200 μ l) for 48 h prior to analysis. Aliquots (25 μ l) of each condition were transferred to a LIA-plate (Greiner), the

CellTiter-Glo reagent (50 µl) was added and luminescence was read after a 10 min incubation using a Synergy H1 plate reader at 27 °C. Data were collected in triplicate and were fitted by GraphPad Prism using the log(inhibitor) vs. response – variable slope (four parameters) equation.

Generation of the *gmps* conditional null cell line—The *gmps* conditional knock out cell line (c-null) was constructed using the fusion PCR method (Nguyen *et al.*, 2013, Merritt & Stuart, 2013). First a Tet-regulated expression construct for *Leishmania major* GMPS (*LmGMPS*) was generated by cloning the PCR amplified *L. major gmps* gene (primers 11 and 12 in a PCR reaction with *L. major* genomic DNA (a gift from Buddy Ullman)) into the pLew100v5 vector (gift from George Cross). The *L. major gmps* gene was used because of difficulties in cloning the *T. brucei gmps* gene into the pLew100v5 vector in the sense direction. The NotI linearized vector was transfected into SM cells and selected using phleomycin. Knockout constructs to replace the two alleles of the *gmps* gene with the hygromycin (*hyg*) and puromycin (*pac*) resistance genes were generated by fusion PCR. The *gmps* 5' and 3' UTRs were amplified from *T. brucei* SM genomic DNA using *gmps* specific primers (primers 1-4); the 5' UTR reverse primer included an overhang complementing the *hyg* or *pac* resistance gene and the 3' UTR the forward primer included an overlap to the resistance gene (primers are listed in Table 1). The *hyg* or *pac* resistance genes were also amplified by PCR (primer pairs 7/8 (*hyg*) or 15/16 (*pac*), Table 1) from plasmid pLew90 (gift from George A.M. Cross (Wirtz *et al.*, 1999)) or pHD1437 (gift from Christine Clayton lab), respectively. Fragments were then used as template for fusion PCR with primers 5 and 6 (Table 1) targeting the internal regions of the 5' and 3' UTRs. The resulting fusion product (600 ng) containing the *hyg* selection marker was transfected into the SM cells containing the *LmGMPS* Tet regulated expression construct in the presence of Tet and recombinants (conditional single knockout line *gmps*(+/-)) were selected using hygromycin. PCR was performed to corroborate efficient allele replacement with the *hyg* resistance gene, using primers 1 and 8 (Table 1). The GMPS c-null cell line was created by transfection of the conditional single knockout cells with the fusion product containing the *pac* selection marker (above) and recombinants were selected with puromycin (0.1 µg/mL), in medium also containing hygromycin and Tet. PCR was performed to confirm that the resulting *TbGMPS* c-null cell line had incorporated both selection markers (primers 5/16 and 5/8) and no longer contained a copy of the *T. brucei gmps* gene (primers 5/6). *T. brucei* BSF cells were cultured in HMI-11 medium throughout the selection process to isolate the *TbGMPS* c-null cell line.

Generation of the *T. brucei gmps* null cell line—The *gmps* null cell line was created by replacing the two alleles of the *gmps* gene with the drug resistance markers for *hyg* and blasticidin (*bsd*). The *gmps* 5' and 3' UTRs, *hyg* and *bsd* genes (primers Table 1) were amplified as described above (the *bsd* resistance gene was amplified from pLew300 vector (Willert & Phillips, 2008)). The resulting fusion PCR product containing *hyg* was transfected into SM cells grown in HMI-19 medium containing guanine (100 µM) and was selected using hygromycin to generate *gmps* single knockout cells. This line was then subsequently transfected with the *bsd* fusion PCR product and selected using blasticidin to generate the *gmps* null cell line. PCR was used to confirm that the two *gmps* alleles were

replaced with the *hyg* and *bsd* resistance cassettes using primer pairs 5/8 and 5/26 and no longer contained a copy of the *T. brucei gmps* gene (primers 5/6) (Table1).

***In vivo* viability analysis of the *T. brucei gmps* null cell line in mice**

C57BL/6J were purchased from the Wakeland Laboratory (UTSW). Two groups of C57BL/6J mice (8 weeks old, $n = 4$) were infected intraperitoneally with 10^3 wild-type (SM) or *gmps* null *T. brucei* parasites. Before infection, these parasites were cultured in HMI-19 medium plus 100 μ M guanine (without antibiotics) and washed three times with HMI-19 medium alone before injection. Mice were monitored every day for general appearance and behavior. Parasitemia was monitored daily from the third day post-infection onwards, collecting 1 μ l of blood from the tail in a 1:150 dilution in medium and counting in a hemocytometer under a microscope. Both parasitemia and mice survival were monitored over a period of 30 days.

Quantitation of nucleotide pools in *T. brucei* BSF cells LC-MS/MS analysis

T. brucei SM and GMPS c-null cells (50 ml cultures) were grown in HMI-19 medium, in the presence or absence of Tet (1 μ g/mL daily) to a density of 1.5×10^6 cells/mL. Cells were pelleted by centrifugation at 3000 g and washed once with cold PBS. Washed cell pellets were suspended in 2 ml 65% methanol and frozen in liquid nitrogen. Cell metabolites were extracted by 10 cycles of freeze/thawing followed by centrifugation at 10,000 rpm for 10 min. Supernatant (1 ml) was collected, vacuum dried, and stored at -80°C . The supernatant was dried by vacuum dryer for 3 h, re-suspended in formic acid buffer (0.1% in HPLC grade water) and filtered through a 0.2 micron centrifuge filters (Grace Davison). Nucleotide metabolite analysis was performed by LC-MS/MS on a Shimadzu Prominence LC20/SIL-20AC HPLC coupled to a ABSCIEX 3200 QTRAP triple quadrupole mass spectrometer as described (Tu *et al.*, 2007). Chromatographic separation was performed using a C18-based column with polar embedded groups (Synergi Fusion, 150×2.0 mm 4 μ , Phenomenex). Infusion quantitative optimization was performed to acquire optimal product ion mass for each metabolite. Multiple reaction monitoring (MRM) was used to detect and quantitate metabolites. The two most abundant daughter ions were used when possible and metabolite peak area was normalized to total ion content. Daughter ion pairs were as follows: GMP (364/152 and 364/135), GDP (443.9/152.1 and 443.9/135), GTP (521.9/78.9), AMP (348/136 and 348/119), ADP (428/136 and 428/119), ATP (508/136.2 and 508/410.1), XMP (364.9/97.1 and 364.9/152.9) and IMP (349/137). Data were plotted and analyzed (unpaired t-tests with equal SD) using GraphPad Prism.

Quantitation of ATP by a luciferase-based assay

GMPS cDKO cells were grown in HMI-19 media, in the presence or absence of Tet (1 μ g/mL daily) to a density of $1.5\text{--}2.0 \times 10^6$ cells/mL. Cell numbers were quantitated by hemocytometer and a 2-fold serial dilution of cells from 3×10^4 – 1×10^6 cells/ml was made to demonstrate a linear relationship between cell number and luminescence. Aliquots of each (50 μ l) were mixed with CellTiter-Glo reagent (50 μ l) and luminescence was read after a 10 min incubation using a Synergy H1 plate reader at 27°C . Luminescence values

were normalized per cell number and the data shown are for analysis of 500,000 cells. Data were collected in biological triplicate.

Molecular modeling and sequence alignment

The program PyMOL (*Pymol Molecular Graphics System* (2000) DeLano Scientific, San Carlos, CA) was used to generate all structure figures using coordinates for human (2VXO) and *E. coli* (1GPM) GMPS. Sequences were aligned using Clustal Omega (1.2.1) multiple sequence alignment software as described in Fig. 1SA.

General Methods

RNA and DNA purification

Genomic DNA was isolated from 1×10^7 *T. brucei* cells using the DNAzol extraction method as recommended by the manufacture (Molecular Research Center, Inc). RNA was extracted from cells ($\sim 3 \times 10^8$) collected at different induction times and total RNA was extracted using TRIzol® (Invitrogen), according to manufacturer's protocol. Then, mRNA was purified from 250 µg total RNA using the Dynabeads® mRNA purification kit (Invitrogen).

mRNA quantitation by RT-PCR

Quantitative real-time PCR was performed using SYBR Green Mastermix (Bio-Rad). Cultures containing 5×10^7 cells, grown with or without tetracycline (1 µg/mL daily), were pelleted and washed in PBS once and suspended in 1 ml Trizol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's instructions followed by quantification via spectrophotometer (BioTek Hybrid Plate Reader). 5 µg total RNA was treated with 1 unit DNaseI (Invitrogen) and RT-PCR was performed (Invitrogen Superscript II RT-PCR Kit). *Lm*GMPS RNA abundance was quantified with primer pairs 21/22 or 23/24 respectively (Table 1) using iTaq SYBR Green Supermix with ROX (BioRad) in a reaction (20 µl) containing 50 ng RNA, 1× Supermix, and 7.5 pmols of each primer. The amplification conditions were: 95 °C for 15 s, 60 °C for 1 min, and data collection for 40 cycles. Relative gene abundance was calculated by the $\Delta\Delta C_t$ method (Winer *et al.*, 1999) using either DHODH or TERT (Brenndorfer & Boshart, 2010) as the reference gene (primer pairs 21/22 or 23/24 respectively (Table 1)).

Western blot analysis

Parasites ($\sim 10^8$) were harvested by centrifugation (3000 rpm, 10 min) and washed 3× with cold (4°C) phosphate-buffered saline (PBS) (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Pellets were resuspended in protein lysis buffer (50 mM HEPES, pH 8.0, 100 mM NaCl, 5 mM β-mercaptoethanol, and protease inhibitor cocktail (as above)). Cells were lysed by three freeze/thaw cycles and clarified by high-speed centrifugation. Protein concentration was determined using the Bradford assay (Bradford, 1976). Protein (20 µg total protein per well), was separated by a 12% SDS-PAGE gel and transferred to a PVDF membrane (iBlot® Transfer Stack, PVDF, Invitrogen) using a dry transfer iBlot® Gel Transfer Device (Invitrogen) for 7 min. Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline (TBS) (20 mM Tris-HCl pH 7.6, 137 mM NaCl, pH

7.6) 2 h at RT. Primary rabbit *TbGMPS* polyclonal antibodies were generated by a commercial vendor (COVANCE) from purified recombinant *TbGMPS* (tag removed by the protease TEV as described (Grishin *et al.*, 1996)). Dilutions for primary antibodies in 5% milk in TBS-T (TBS plus 0.05% Tween) were α *TbGMPS* – 1:2000. Secondary antibodies (anti-rabbit), were used at 1:10,000 dilution in 5% nonfat milk TBS-T. Dihydroorotate dehydrogenase levels were measured as a loading control using the previously described antibody (1:2500 dilution)(Arakaki *et al.*, 2008). Protein was detected by SuperSignal West Femto Chemiluminescent Substrate (Thermo scientific.) according to the manufacturer. Bands were visualized by ImageQuant LAS 4000 (GE).

Flow cytometry

For cell cycle examination, cells were grown to a density of 10^6 cells/mL in 20 ml HMI-11 medium, pelleted by centrifuged, resuspended in 70% ethanol and incubated at 4 °C overnight. Cells were washed using 10 ml of cold PBS, resuspended in 1 ml of PBS containing 10 μ g/ml propidium iodide (PI) (Sigma), 10 μ g/ml RNase A, and incubated at RT for 20 - 30 mins. Flow cytometry was performed with FACSCalibur (BD Biosciences) using the FL2-H and side-scatter detectors. A total of 10000 gated events were collected for each sample. Data was analyzed using software FlowJo (NIH).

DNA staining of trypanosomes

Cells were grown to a density of up to 1×10^6 cells/ml in HMI-12 medium, collected by centrifugation, washed in PBS and resuspended in 1 ml PBS. Cell suspension (0.2 – 0.3 ml) was placed on a cover slip for 20 min, the cover slip was then placed cell side up in a well of a 6-well plate, 1 ml 4% paraformaldehyde (PFM) (16% paraformaldehyde stock from Electric Microscopy Sciences) was added and cells were incubated for 30 min at RT. PFM was removed by vacuum and the cover slip was washed with 1 ml of cold PBS 2 $\times$, followed by a wash with PBS plus 0.1% triton X-100, and finally three 1 min washes with PBS. Cover slips were removed from the well and mounting medium (anti-fade, Invitrogen containing 4', 6-diamidino-2-phenylindole (DAPI)) (10 μ l) was dropped onto the slide and the coverslip with cells was mounted on the slide. Cells were visualized using Deltavision Deconvolution, AppliedPrecision. NL.5 120 microscope. The differential interference contrast (DIC) and fluorescence field images were obtained at the same time. Images were visualized using the Image J software (NIH). For manual counting of nucleus and kinetoplastid content, 100 cells were counted for each condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health grants 1R01AI078962 (to KS and MAP), 2R37AI034432 (to MAP) and GM007062 (to CL), the Welch Foundation grant I-1257 (to MAP) and Fundação para a Ciência e Tecnologia (FCT, Portugal) SFRH/BD/51286/2010 to FRF. MAP holds the Beatrice and Miguel Elias Distinguished Chair in Biomedical Science and the Carolyn R. Bacon Professorship in Medical Science and Education. The authors would like to thank Drs. Joe S. Takahashi (HHMI, UT Southwestern) and Luisa M. Figueiredo (Universidade de Lisboa) for help with the *in vivo* essentiality studies, and Yunyun Zhou for helpful discussions on statistical analysis.

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Abbreviations

GMPS	GMP synthase
GMPR	GMP reductase

GDA	guanine deaminase
IMPDH	inosine-5'-monophosphate dehydrogenase
ADSS	adenylosuccinate synthase
AK	adenosine kinase
AD	AMP deaminase
ADSL	adenylosuccinate lyase
NH	inosine-uridine (or non-specific) nucleoside hydrolase
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
XPRT	xanthine phosphoribosyltransferase
APRT	adenine phosphoribosyltransferase
DON	6-Diazo-5-oxo-L-norleucine
TERT	telomerase reverse transcriptase
null	gmps (-/-) cells
c-null	<i>gmps</i> (-/-) null cells containing a Tet regulated copy of <i>Leishmania gmps</i>
BSF	<i>T. brucei</i> bloodstream form cells

Fig. 1. Purine salvage pathway in *T. brucei*

A. Overall pathway. GMPS, GMP synthase; GMPR, GMP reductase; GDA, guanine deaminase; IMPDH, inosine-5'-monophosphate dehydrogenase; ADSS, adenylosuccinate synthetase; AK adenosine kinase; AD, AMP deaminase; ADSL, adenylosuccinate lyase; NH, inosine-uridine (or non-specific) nucleoside hydrolase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase. Druggability scores are shown in parenthesis and were obtained from tdrtargets.org. Scores of >0.6 (range 0 – 1) are considered to be a strong indication that a target is “druggable”, e.g. likely to be capable of binding drug-like molecules with good affinity (Aguero et al., 2008). B. Enzymatic reaction catalyzed by GMPS. R5P, ribose 5'-phosphate.

Fig. 2. Effects of conditional GMPS knockdown (*gmps* c-null cells) on *T. brucei* bloodstream form cell growth

A. Analysis of the c-null cell line grown in HMI-11 medium in the presence or absence of added purine bases before (Control, day 0) or after withdrawal of Tet from the medium for the indicated time where G, guanine, X, xanthine. B. Representative qPCR analysis of c-null cells. Cells were grown $\pm$ Tet for 0, 1 or 6 days in HMI-11. Expression levels of GMPS mRNA are reported relative to either the TERT or DHODH reference gene. C. Analysis of the c-null cell line grown in HMI-19 medium in the presence or absence of added bases (concentration shown in μM) before (Control) or after withdrawal of Tet from the medium for the indicated time. D. Cell growth effects of guanine supplementation (0.8 – 100 μM) on c-null cells 4 days after Tet withdrawal for cells cultivated in either HMI-11 or HMI-19 medium. E. Cell growth effects of hypoxanthine supplementation (1.0 – 1000 μM) in HMI-19 medium in the presence or absence of guanine (100 μM) 4 days after Tet withdrawal. Total cell number is shown and represents cell number multiplied by the dilution factor relative to day zero. All data represent the mean of three biological replicates.

Fig. 3. Effects of GMPS knockout on *in vitro* growth and viability in a mouse model of infection

A. GMPS null cells were grown in HMI-19 medium either in the absence or presence of supplementation with the indicated purine base (concentration is shown in μM) for control wild-type (WT) (green squares) and *gmps* null cells (white squares) cells. G, guanine, X, xanthine, A, adenine, H, hypoxanthine. Data represent the mean and standard deviation for three independent biological replicates. Inset: Western blot analysis (20 μg total protein/lane) of wild-type (WT) and *gmps* null cells grown in the presence of guanine (100 μM). Blots were probed with either *T. brucei* GMPS antibody or DHODH antibody as a loading control. B. Mice were intraperitoneally infected with 1000 WT (SM) control or *gmps* null parasites and parasitemia (parasites/mL of blood) assessed daily. Data represent the mean plus standard deviation for 4 mice per condition. C. Mouse survival curve versus days post-infection with either WT or *gmps* null cells. Median WT-infected mice survival is 5.5 days post-infection.

Fig. 4. Morphology and cell cycle analysis of *T. brucei* blood form *gmps* null and c-null cells
 A. Flow cytometry analysis of null cells grown with guanine (100 μ M). B. Flow cytometry analysis of cells 24 h after guanine withdrawal. Cells were stained with propidium iodide (PI). PI fluorescence (FL3-Area) is plotted versus cell count. C. Images of representative cells before and 24 h after guanine withdrawal where panel 1 is an overlay of DAPI and DIC images, panel 2 is DAPI staining and panel 3 shows the DIC image. D. Flow cytometry analysis of cells prior to Tet withdrawal (day 0). E. Flow cytometry analysis of cells 48 h after Tet withdrawal. F. Images of representative cells before and 48 h after Tet withdrawal (2D).

Fig. 5. Nucleotide quantitation of the effects of GMPS knockdown in c-null cells

Intracellular levels of guanine nucleotides detected by LC-MS/MS for cells collected before and 2 days after Tet removal leading to loss of GMPS expression showing A) GMP and GDP levels, B) GTP levels for a representative data set. Relative ion abundance is shown. The data set includes three independent biological replicates collected on the same day. See Fig. S1 for data on additional nucleotides. P values are displayed where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0005$, **** $P < 0.0001$ for one-tailed t-test. C. Ratio of -Tet/+Tet ion abundance is plotted for the indicated nucleotide. Data represent the mean of four - six independent biological replicates that were collected on three separate dates. Error bars represent the standard deviation of the mean. MRMs to detect the indicated nucleotides were as follows: GMP (364/152), GDP (444/152), GTP (524/152), IMP (349/137), XMP (365/97), AMP (348/136), ADP (428/136).

Table 1
***T. brucei* culture medium purine base and nucleoside concentrations in comparison to human plasma and CSF**

Nucleoside	HMI-11 [*] -FCS	HMI-19 [*] -FCS	CMM ^{**} +FCS	Human plasma ^{***}	Human CSF ^{***}
Hypoxanthine	1 mM	0	4.4 µM	8 µM	3.4 µM
Thymidine	0.16 mM	0	ND	0.2 µM	0.06 µM
Guanine	0	0	nd	ND	nd
Guanosine	0	0	nd	0.8 µM	nd
Inosine	0	0	nd	1.4 µM	0.62 µM
Xanthine	0	0	nd	1.4 µM	9 µM
Adenine	0	0	nd	0.5 µM	0 – 0.2
Adenosine	0	0	nd	1.0 µM	0 - 0.1

^{*} Data represent concentrations prior to addition of 10% fetal bovine serum (FBS).

^{**} Data were taken from (Creek et al., 2013) and were determined by LC/MS analysis after the addition of 10% FBS;

^{***} data taken from www.hmdb.ca and are average values of reported data. Human plasma purine base and nucleoside levels are also reported in (Boulieu et al., 1983; Hartwick et al., 1979; Slowiaczek & Tattersall, 1982). ND, not detected; nd, not determined.

Table 2Recombinant *T. brucei* GMPS steady state kinetic analysis.

	K_{mapp} (μM)			k_{cat} s⁻¹
	XMP	ATP	Glutamine	
<i>T. brucei</i>	8.8 ± 0.11	200 ± 21	240 ± 11	4.7 ± 0.07
<i>P. falciparum</i> [*]	17	260	470	0.43
Human ^{**}	43	160	440	5.3

k_{cat} is the average value calculated for the three different substrate titration curves.

^{*} data taken from (Bhat et al., 2008);

^{**} data taken from (Lou et al., 1995)

Table 3
Inhibition of parasite growth and recombinant *Tb*GMPS by Acivicin and DON

	IC _{50,App} <i>Tb</i> GMPS*	EC ₅₀ WT cells	EC ₅₀ <i>gmps</i> null cells
	μM		
Acivicin	26 (22 – 30)	1.3 (1.2 – 1.5)	1.8 (1.6 – 2.0)
DON	nd	2.6 (2.2 – 3.1)	3.0 (2.5 – 3.8)

* The time dependence of the enzyme inhibition was not evaluated and thus the IC₅₀ values are apparent and dependent on the assay time. *T. brucei* BSF WT (SM) and GMPS null cells were grown in the presence of 100 μM guanine in HMI-19 medium. Error in parenthesis represents the standard error of the fit for triplicate data. nd, not determined.